Cohesion-Defective Mutants of *Myxococcus xanthus*

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Cohesion of *Myxococcus xanthus* cells involves interaction of a cell surface cohesin with a component of the extracellular matrix. In this work, two previously isolated cohesion-defective (*fbd*) mutants were characterized. The *fbdA* and *fbdB* genes do not encode the cohesins but are necessary for their production. Both mutants produce type IV pili, suggesting that PilA is not a major cohesin.

Myxococcus xanthus cells foster social behaviors like social (S) motility and fruiting body development through cohesive interactions between cells. *M. xanthus* has two motility motors; adventurous (A) motility allows the movement of individual cells, and S motility facilitates the movement of groups of cells in swarms (10). S motility depends on type IV pili (TFP) (29, 31, 32), as well as an extracellular matrix (ECM) composed of exopolysaccharide and protein (1, 34). The exopolysaccharide portion of the ECM provides the anchor for TFP, which pull the cells forward by pilus retraction (19, 23). There are probably other cohesive interactions since pilus-deficient mutants form fruiting bodies in which cells and spores are cohesive.

Mutations in the *dsp* (dispersed growth) locus, also known as the *dif* (defective in fruiting) locus, result in the loss of ECM, S motility, agglutination, and development (18, 25, 33). A ligand-receptor interaction may be responsible for cohesion since *dsp/dif* mutants can agglutinate when they are mixed with ECM-producing cells or ECM purified from *M. xanthus* (1, 6, 25). In an effort to understand the interaction between ECM and cells, candidate receptor mutants (*fbd* [fibril binding-defective] mutants) were identified in a *dsp* background by the fact that they could not be agglutinated with isolated ECM (7). While the *fbd* mutant phenotype is consistent with loss of the cell surface cohesin, the mutant alleles were never examined in a wild-type background. The purpose of this work was to move the *fbd* alleles into the wild-type genetic background, examine the *fbd* phenotypes, identify the disrupted loci, and determine if candidate cell surface cohesins were encoded.

Strains LS2225 (*fbd1025*) and LS2226 (*fbd1026*) were constructed by Mx4-mediated transduction of the Tn5 insertions in MD1025 (*dsp1680 fbd1025*) and MD1026 (*dsp1680 fbd1026*), respectively, into wild-type strain DK1622 (5) (Table 1 shows the strains used in this study). LS2225 does not form mature fruiting bodies and produces only 0.3% of the viable spores produced by the wild type (Fig. 1A). The fact that LS2225 produces so few viable spores contrasts with the production of spores by MD1025, which produces 80% of the viable spores produced

by the wild type (data not shown). Thus, *fbd1025* inhibits sporulation in wild-type cells, but it may suppress the sporulation defect of *dsp1680* cells, assuming that MD1025 does not contain another mutation. LS2226 does not form mature fruiting bodies, but it produces a lawn of spores (33% of the viable spores produced by the wild type) (Fig. 1A). The fruiting body formation and sporulation phenotypes of LS2226 are similar to those of MD1026 (data not shown).

Defects in A or S motility can be determined by looking at the colony morphology of double mutants containing a known A- or S-motility mutation. If a mutation prevents S motility, nonmotile colonies are formed when the mutation is moved into an A[−] strain. Genomic DNAs from LS2225 and LS2226 were transformed into DK10410 (*pilA*; A⁺ S[−]) and MxH1777 (*aglU*; A[−] S⁺) as described previously (27). DK1622 formed large spreading colonies that had a morphology characteristic of A⁺ S⁺ colonies (Fig. 1B). Both LS2225 and LS2226 formed small spreading colonies that were typical of an A⁺ S[−] phenotype (Fig. 1B). The colony morphologies of LS2225 and LS2226 were similar to that of DK10410, which was A⁺ S[−] due to loss of the TFP structural protein, PilA (29). LS2209 (*pilA fbd1025*) and LS2211 (*pilA fbd1026*) formed motile colonies in spite of the S-motility mutation, indicating that both of these mutants maintained A motility (Fig. 1B). MxH1777 was an A-motility mutant and formed large spreading colonies with smooth edges, which is characteristic of A[−] S⁺ colonies (28). As expected, LS2210 (*aglU fbd1025*) formed nonmotile colonies, confirming that *fbd1025* eliminates S motility (Fig. 1B). However, LS2212 (*aglU fbd1026*) formed spreading colonies characteristic of S motility (Fig. 1B). These results suggest that *fbd1026* at least partially suppresses S motility in wild-type cells. Although this phenotype is rare, it is not unique, as deletion of either the entire *che4* chemosensory system or *cheY4* produces a similar but less dramatic phenotype; enhanced swarming is observed in a strain lacking A motility compared to the swarming of wild-type cells with the same deletions (27). LS2212 produced ECM (see below) and fruiting bodies (data not shown), suggesting that inhibition of S motility in a wild-type background may be due to reduced ECM production.

The cohesive properties of LS2225 and LS2226 were examined in order to determine if the *fbd* mutations in a wild-type background eliminate cohesion, as observed previously for the *dsp1680 fbd* mutants (7). Wild-type cells placed in cohesion

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TABLE 1. Bacterial strains used in this study

Strain	Description or genotype	Reference or source
DK1622	Wild type	13
LS2225	Mx4 (MD1025) × DK1622; Km ^r	This study
LS2226	Mx4 (MD1026) × DK1622; Km ^r	This study
DK3468	<i>dsp1680</i>	25
MD1025	<i>dsp1680 fbd1025</i>	7
MD1026	<i>dsp1680 fbd1026</i>	7
DK10410	<i>pilA</i>	Kaiser Lab
LS2209	Mx4 (MD1025) × DK10410; Km ^r	This study
LS2211	LS2226 × DK10410; Km ^r	This study
MxH1777	<i>aglU</i>	28
LS2210	Mx4 (MD1025) × MxH1777; Km ^r	This study
LS2212	LS2226 × MxH1777; Km ^r	This study
LS1102	<i>stk1907</i>	8
LS2231	Mx4 (MD1025) × LS1102; Km ^r	This study
LS2213	Mx4 (LS2226) × LS1102; Km ^r	This study

buffer rapidly agglutinated; within 45 min less than 5% of the cells remained in suspension (Fig. 2A). In contrast, after 120 min more than 90% of the LS2225 and LS2226 cells remained in suspension (Fig. 2A), and after 24 h more than 80% of the cells remained in suspension (data not shown). In order to determine whether LS2225 and LS2226 can be agglutinated by wild-type cells, each strain was mixed at a 1:1 ratio with DK1622 (Fig. 2B). After 180 min, when less than 10% of the DK1622 cells remained in suspension, only about 50% of the mixed cell suspensions agglutinated, indicating that the *fbd* mutants are not cohesive. These results indicate that both LS2225 and LS2226 are defective for the production of cohesive ligands.

The ECM is composed of polysaccharide and protein. Immunoblot analysis did not detect the ECM protein FibA (13) (Fig. 3A). Both Congo red and trypan blue bind the ECM polysaccharide (2, 8). Trypan blue binding assays were performed in triplicate with 10 mg/ml trypan blue for 30 min at room temperature. While DK1622 bound $15.6\% \pm 1.8\%$ of the dye, LS2225 bound $2.1\% \pm 0.4\%$ and LS2226 bound $0.5\% \pm 0.5\%$, indicating that there was a deficiency in exopolysaccharide production, which was confirmed with a Congo red dye-binding assay (data not shown). Interestingly, LS2212 (*aglU fbd1026*) bound $28.9\% \pm 3.5\%$ of the dye, indicating that LS2212 overproduces ECM, which is consistent with the hypothesis that the restoration of S motility may be due to the restoration of ECM production.

One potential cohesin is the pilin monomer PilA, which binds ECM polysaccharide (19, 23). Pilin production was examined in the *fbd* mutants by immunoblot analysis of whole cells using an antibody against PilA (Fig. 3B). LS2225 and LS2226 produced PilA, albeit at reduced levels compared to wild-type cells. LS2225 and LS2226 produced reduced, but significant levels of surface pili compared to the wild type based on an immunoblot of pili sheared from the cell surface (Fig. 3C). These results suggest that the presence of pili is not sufficient for agglutination of the *fbd* mutants by wild-type cells.

Genomic DNA from each strain was digested with SalI and cloned into pUC19 with selection for kanamycin resistance, the marker in the Tn5 insertion causing the mutation. The plasmid

inserts were sequenced, and the DNA sequences were compared with the *M. xanthus* genome (TIGR database [http://cmr.tigr.org/tigr-scripts/CMR/CMrHomePage.cgi]). The site of Tn5 insertion in MD1027 (7), a *dsp1680 fbd1027* mutant, was also determined.

The gene disrupted in LS2225 corresponds to MXAN6699 in the *M. xanthus* DK1622 genome (TIGR database [http://cmr.tigr.org/tigr-scripts/CMR/CMrHomePage.cgi]) and is referred to below as *fbdA* (Fig. 4A). The site of transposon insertion in

FIG. 1. Fruiting body formation, sporulation efficiency, and colony spreading. The strains and their genotypes are indicated above the photographs. (A) Fruiting body formation after 5 days on TPM agar (10 mM Tris HCl [pH 7.6], 8 mM MgSO₄, 1 mM K₂HPO₄-KH₂PO₄ [pH 7.6], 1.5% agar [Difco]). Scale bars = 500 μm. The number of spores and percentage of sporulation compared to the wild-type sporulation are indicated under each panel. (B) Colony spreading after 5 days on CYE agar [10 g of Difco Casitone per liter, 5 g of yeast extract per liter, 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS; pH 7.6), 4 mM MgSO₄, 1.5% agar (Difco)]. Scale bars = 5 mm.

FIG. 2. Cellular cohesion assays. (A) Cells were grown in CYE medium and washed in 10 mM MOPS, and 1×10^9 cells were resuspended in cohesion buffer (10 mM MOPS, 1 mM MgSO_4 , 1 mM CaCl_2). Turbidity was determined every 15 min for 2 h. The strains used were DK1622 (■), LS2225 (●), and LS2226 (▲). (B) Assays performed as described above except that preparations were incubated for 3 h. The strains used were DK1622 (■) and 1:1 mixtures of DK1622 and LS2225 (●) and of DK1622 and LS2226 (▲).

MD1027 is just upstream of *fbdA* (Fig. 4A). MXAN6699 is upstream of the *dif* locus, which encodes a chemosensory system that regulates matrix production and lipid chemotaxis (2, 4) (Fig. 4A). *fbdA* is predicted to encode a protein homologous to DUF1111 (a protein with an unknown function; 2e-178) and COG3488 (a predicted thiol oxidoreductase; 1e-112). None of the proteins in these families have been experimentally characterized. The amino acid sequence is predicted to contain two *c*-type cytochrome superfamily profiles. *c*-Type cytochrome superfamily profiles are found in all bacterial cytochromes and in the active site of many periplasmic heme-binding enzymes, including fatty acid *cis-trans* isomerase (12), diheme cytochrome *c* peroxidases (9), and one type of alcohol dehydrogenase (26). The amino acid sequence is predicted to contain a signal peptide, suggesting that FbdA may be a periplasmic protein. One possibility is that *fbdA* encodes an enzyme involved in producing a signal that stimulates matrix production. It has been suggested previously that an unidentified signal for matrix production is recognized by the Dif chemosensory sys-

FIG. 3. Immunoblot analysis of the *fbd* mutants. (A) Immunoblot analysis of the ECM protein FibA. Whole-cell lysates were prepared from 5×10^7 cells, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and probed with polyclonal anti-FibA generated against an internal mitogen-activated protein peptide. The positions of molecular mass standards (in kDa) are indicated on the left. The strains used are indicated at the top. (B) Immunoblot analysis for the pilin protein, PilA. Whole-cell lysates were prepared from 5×10^7 cells, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and probed with anti-PilA (30). The position of a molecular mass standard (in kDa) is indicated on the left. The strains used are indicated at the top. (C) Immunoblot of pili prepared by shearing (30). A total of 2.5×10^9 cells were resuspended in TPM buffer and vortexed for 3 min at the maximum speed. Cells were removed by centrifugation. Pili were precipitated by addition of MgCl_2 to a final concentration of 100 mM and incubation on ice for 1 h. Pili were collected by centrifugation at 4°C . The pili from 5×10^8 cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with anti-PilA. The position of a molecular mass standard (in kDa) is indicated on the left. The strains used are indicated at the top.

tem (2, 4). A secondary mutation in the *stk1907* locus has been shown to restore ECM production and social behavior to most S-motility mutants except *dsp* mutants (8). LS2231 (*stk1907 fbdA*) is restored for ECM production, FibA production, cohesion, and fruiting body formation (data not shown). This

FIG. 4. Genomic region surrounding *fbdA* (A) and *fbdB* (B). The *fbd* genes are indicated by arrows with diagonal lines. The sites of transposon insertion are indicated by arrowheads and are designated by Ω followed by the allele number. The *dif* locus is indicated by gray arrows. Genes predicted to encode homologs of characterized proteins are indicated by black arrows. Genes predicted to encode hypothetical proteins are indicated by open arrows.

result suggests that FbdA is not essential for synthesis, transport, or assembly of the ECM, which is consistent with a defect in signal generation.

The gene disrupted in LS2226 corresponds to MXAN6307 and is referred to below as *fbdB* (Fig. 4B). *fbdB* is the last gene in a cluster encoding a putative M50 peptidase and a putative fatty acid desaturase. FbdB is homologous to pfam00654 (voltage gated chloride channel; 2e-32) and COG0038 (EriC, a chloride channel protein; 4e-45). While it is not clear how a putative chloride channel could regulate ECM production and S motility, an intriguing possibility is that osmoregulation may play a role. Osmoregulation is important for fruiting body formation and spore production in *M. xanthus* and is mediated by both MokaA, a hybrid histidine kinase-response regulator, and CyaA, a eukaryote-like cAMP-dependent protein kinase A (14–17). In fungi, protein kinase A mediates a general stress response that is observed during heat shock, oxidative stress, and osmotic stress (20, 22, 24). Osmotic responses of fungi are also mediated by mitogen-activated protein kinase pathways that control growth and morphogenesis, as well as osmoregulation (3, 11). *M. xanthus* possesses a network of signal transduction systems, including two-component systems, serine/threonine and tyrosine kinases, and GTP-binding proteins (TIGR database [http://cmr.tigr.org/tigr-scripts/CMR/CMrHomePage.cgi]), many of which have complex roles in modulating *M. xanthus* social behaviors.

In this work we identified two *fbd* genes that are required for ECM production, cohesion, and fruiting body formation. The lack of trypan blue binding and FibA production by the *fbd* mutants suggests that both FbdA and FbdB are required for ECM production. The fact that these strains could not be agglutinated by wild-type cells suggests that both Fbd proteins are also required for ECM binding. Furthermore, FbdA and FbdB are not likely to be cell surface exposed. Therefore, it seems unlikely that the Fbd proteins function solely as ECM receptors. In both *fbd* mutants, secondary mutations (*stk fbdA* and *aglU fbdB*) restore ECM production, indicating that neither *fbdA* nor *fbdB* is essential for the synthesis, transport, or assembly of the ECM. Together, the data suggest that the *fbd* genes do not encode the cohesins but regulate the production of both the cell surface cohesin(s) and ECM.

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